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Impaired Coordination of Nutrient Intake and Substrate Oxidation in Melanocortin-4 Receptor Knockout Mice

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Mutations in the melanocortin-4 receptor (MC4R) are associated with obesity. The obesity syndrome observed in humans with MC4R haploinsufficiency is similar to that observed in MC4R knockout mice, including increased longitudinal growth, hyperphagia, and fasting hyperinsulinemia. For comparison with other commonly investigated models of obesity and insulin resistance, we have backcrossed *Mc4r*−/− mice into the C57BL/6J (B6) background. Female obese *Mc4r*−/− mice exhibit reduced energy expenditure and an attenuated increase in fatty acid (FA) oxidation after exposure to high-fat diets compared with obese *Lep*^{ob}/*Lep*^{ob} mice. The reduced energy expenditure and FA oxidation correlates with changes in hepatic gene expression. The expression of genes involved in FA oxidation increased in obese *Lep*^{ob}/*Lep*^{ob} mice compared with wild-type and obese *Mc4r*−/− mice. In contrast, a key lipogenic enzyme, FA synthase (FAS), is increased in obese *Mc4r*−/− mice compared with obese *Lep*^{ob}/*Lep*^{ob} mice. Hyperinsulinemia, increased FAS mRNA expression and hepatic steatosis appear to be secondary to obesity in B6 *Mc4r*−/− mice. However, *Mc4r*−/− mice in a mixed genetic background develop severe hepatic steatosis at an early age. This might suggest an important role of the MC4R in regulating liver FA metabolism that is masked on the B6 background. Interestingly, the 10- to 20-fold increase in liver triglyceride in the outbred strain of *Mc4r*−/− mice is not always associated with fasting hyperinsulinemia or increased FAS mRNA expression. This observation suggests that changes in liver secondary to triglyceride accumulation lead to hyperinsulinemia and increased hepatic FAS expression in *Mc4r*−/− mice. (*Endocrinology* 145: 243–252, 2004)

THE CURRENT EPIDEMIC of obesity and type 2 diabetes mellitus (DM2), if unchecked, will place a considerable strain on the health care system (1, 2). The obesity epidemic is likely due to a combination of genetic and environmental factors (3). Analysis of the development of DM2 in inbred mouse strains, in conjunction with studies of humans, suggest that genetic variation is involved in determining the frequency of obesity and DM2 subjects (3, 4).

A reduction in metabolic rate and fatty acid (FA) oxidation, perhaps associated with low sympathetic nervous activity, are thought to increase the risk of developing obesity and associated pathologies such as diabetes and cardiovascular disease (5). To date, the most commonly found single gene mutation discovered to be associated with obesity occurs in the melanocortin-4 receptor (MC4R) gene (6–10). Although hyperphagia is a significant factor in obesity because of MC4R haploinsufficiency (11), the results of pair-feeding studies in mouse models indicate that, in the adult mouse, metabolic factors might also contribute to the obese phenotype of MC4R-deficient subjects (12).

The MC4R is expressed in areas of the central nervous system that regulate the activity of neuroendocrine and autonomic systems (13–15). Consistent with neuroanatomical evidence supporting a role for the MC4R in regulating autonomic activity, *Mc4r*−/− mice exhibit an attenuated thermogenic response to hyperphagia (16, 17) and to the stimulation of renal sympathetic nervous activity by leptin, insulin, and the nonspecific melanocortin agonist MTII (18). MC4R are also required for the increase in oxygen consumption (VO_2) and suppression of food intake by MTII (19, 20). In the paraventricular nucleus of the hypothalamus, MC4R mRNA is expressed in thyroid-releasing hormone (TRH) (15) and corticotropin-releasing factor (CRF) neurons (21). Activation of MC4R on TRH neurons in the paraventricular nucleus of the hypothalamus stimulates TRH synthesis and increases T_3 and T_4 levels in the circulation (15, 22, 23). Activation of MC4R expression on CRF neurons increases CRF transcription and circulating corticosterone levels (21).

Mc4r−/− mice on a mixed 129;B6 background rapidly developed insulin resistance, with fasting hyperglycemia and hyperinsulinemia (12, 24, 25). We have now backcrossed the null *Mc4r* allele onto the C57BL/6J (B6) background, which will facilitate the comparison of the phenotype of *Mc4r*−/− mice with other transgenic and spontaneous mutant models of DM2. In this article, we describe the results of studies using indirect calorimetry and gene expression analysis to examine metabo-

Abbreviations: ACC, Acetyl-coenzyme A carboxylase; AOX, acyl-coenzyme A oxidase; B6, C57BL/6J; BSw;129, Black Swiss [NIHNTac: NIH(S)-Tyrp1$^+$,Tyr$^+$];129; CPT1a, carnitine palmitoyltransferase; CRF, corticotropin-releasing factor; DGAT, acyl-coenzyme A:diacylglycerol acyltransferase; DM2, type 2 diabetes mellitus; EE, energy expenditure; FA, fatty acid; FAS, fatty acid synthase; FFM, fat-free mass; FM, fat mass; FQ, food quotient; IRS, insulin receptor substrate; MC4R, melanocortin-4 receptor; NEFA, nonesterified fatty acid; NMR, nuclear magnetic resonance; PPAR, peroxisome proliferator receptor; PRCF, percent cumulative frequency; RER, respiratory exchange ratio; SREBP, sterol regulatory element binding protein; TG, triglyceride; TRH, thyroid-releasing hormone; VO_2, oxygen consumption; WT, wild-type.

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lism of *Mc4r*−/− and *Lep*^{ob}/*Lep*^{ob} mice. Mice were continuously housed in the metabolic chambers and fed purified diets. Surprisingly, our data indicate that obese *Mc4r*−/− mice have a lower energy expenditure (EE), adjusted for fat-free mass (FFM), when compared with *Lep*^{ob}/*Lep*^{ob} mice. In older obese mice, the expression of genes involved in FA oxidation, carnitine palmitoyltransferase (CPT1a), and acyl-coenzyme A oxidase (AOX) is increased in liver of *Lep*^{ob}/*Lep*^{ob} mice compared with both lean wild-type (WT) and obese *Mc4r*−/− mice. In contrast, the expression of a key lipogenic gene, FA synthase (FAS), is 3-fold higher in older obese *Mc4r*−/− mice compared with *Lep*^{ob}/*Lep*^{ob} mice. *Mc4r*−/− mice on the B6 background develop hepatic steatosis and insulin resistance similar to that observed in *Lep*^{ob}/*Lep*^{ob} mice, which is secondary to obesity. On a mixed genetic background, *Mc4r*−/− mice develop severe hepatic steatosis before the onset of obesity. This could indicate an important role for the MC4R in regulating liver metabolism that is masked on the B6 background.

Materials and Methods

Experimental animals

All studies were reviewed and approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. In experiment 1, we examined EE and estimated substrate oxidation [respiratory exchange ratio (RER)] in obese female *Mc4r*−/− and *Lep*^{ob}/*Lep*^{ob} mice on the B6 background. All mutant mice were generated using heterozygote parents and were genotyped as previously described (16, 26). *Lep*^{ob}/*Lep*^{ob} mice were derived from a colony of B6.V-*Lep*^{ob} mice purchased from Jackson Laboratories (Bar Harbor, ME). WT refers to B6 mice derived from the *Mc4r*+/− and B6.V-*Lep*^{ob} breeding colonies. Fat mass (FM) and FFM were determined in triplicate by nuclear magnetic resonance (NMR) using a Bruker Mice Minispec NMR Analyzer (Bruker Optics Inc., Billerica, MA).

In experiment 2, we analyzed gene expression in preobese *Mc4r*−/− mice. For this experiment, *Mc4r*−/− mice on two genetic backgrounds were used. We examined gene expression in *Mc4r*−/− mice on the B6 background and in *Mc4r*−/− mice derived from an outbred colony on a Black Swiss [NIHNTac:NIH(S)-Tyrp1^{+},Tyr^{+}];129 background (BSw;129).

Three purified diets using lard and soybean oil as the source of fat were purchased from Research Diets, Inc. (New Brunswick, NJ). The low-fat diet [catalog no. D12450B, 15.9 kJ/g, food quotient (FQ) = 0.925] had 10% kJ from fat, 70% kJ from carbohydrate, and 20% kJ from protein. The high-fat diet (catalog no. D12451, 19.7 kJ/g, FQ = 0.823) had 45% kJ from fat, 35% kJ from carbohydrate, and 20% kJ from protein. The very high-fat diet had 60% kJ from fat and 20% kJ from carbohydrate and protein (catalog no. D12492, 21.8 kJ/g, FQ = 0.781).

Indirect calorimetry

Indirect calorimetry was performed, as described previously, using a 16-chamber Oxymax system (Columbus Instruments, Columbus, OH) (16, 27). Mice were housed on a 12 h light and dark cycle (dark 0100–1300 h, light 1300–0100 h) at 28 C. Mice were allowed 5–7 d to acclimate to the novel environment with free access to food, which was placed on the wire mesh at the bottom of the chamber, and water. Plastic tubing was supplied to minimize stress associated with housing on wire mesh.

EE (kJ/h) was calculated using VO_2 ($VO_2 \times [3.815 + (1.232 \times RER)] \times 4.1868$). Percent energy from substrate oxidation (F%, C%) was estimated using the RER (28), and the balance was then calculated by subtracting total kJ of substrate oxidized from the amount ingested over the 3-d period. RER, VO_2, and EE data were analyzed as bins of either 4 h or as dark and light periods.

Percent relative cumulative frequency (PRCF) curves were calculated as described previously (29). The analysis of PRCF curves is a recently developed method used to evaluate calorimetry data. To calculate the PRCF curves for VO_2, EE, and RER, data sets from mutant or WT mice were pooled, and the cumulative frequency calculated in Microsoft Excel (Microsoft Corporation, Redmond, WA). The advantage of this method is that it allows for the comparison of the range of metabolic data between groups. For VO_2 and EE, this provides an easy visual method for comparing metabolic rate between low (corresponding to resting metabolic rate) and high (activity-based EE) values. Differences in basal metabolic rate, affecting VO_2 and EE throughout the diurnal cycle, are predicted to result in a parallel shift of the S-shaped cumulative frequency curve. On the other hand, differences in activity-based EE would be predicted to affect the curve in the upper quartile only.

Triglyceride (TG), glucose, insulin, and FA measurements. Total lipid content of liver was quantitated using a chloroform-methanol extraction (30), and tissue TG content was determined as described previously (31). Commercially available kits were used to determine serum insulin (CrystalChem Inc., Downers Grove, IL) and TG (GPO-Trinder; Sigma-Aldrich Corp., St. Louis, MO). Venous blood glucose levels were measured from tail-vein blood sample using a Glucometer Elite (Bayer Corp., Elkhart, IN).

RNA expression analysis

Total RNA, extracted from tissues using TRI Reagent, was kept in Rnase-free formazol (Molecular Research Center, Inc., Cincinnati, OH). Quantitation of mRNA expression, using cyclophilin B as a standard, was performed using an ABI Prism 7700 HT sequence detection system (Applied Biosystems, Foster City, CA) as described previously (32). The primers and probes for cyclophilin B and peroxisome proliferator receptor (PPAR) α have been described previously (29, 33). Primer and probe combinations were designed using the genomic sequence obtained from the National Center for Biotechnology Information [gene and accession no.: AOX, AF006688; L-CPT1a, AF17175; FAS, AF127033; FA translocase/CD36, NM007643, sterol regulatory element binding protein (SREBP) 1, AF374266]. All primer-probe combinations were designed to span introns to minimize signals arising from RNA template and genomic DNA contamination.

Gel electrophoresis and immunoblotting

Frozen tissue was homogenized in a buffer containing 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonylfluoride, 1 μM pepstatin, 50 trypsin inhibitory mU of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 min at 5000 rpm to remove any debris and insoluble material and then analyzed for protein content.

Protein extracts were separated in 5%, 7.5%, 10%, or 12% polyacrylamide (acrylamide from National Diagnostics, Atlanta, GA) gels containing sodium dodecyl sulfate (SDS) and transferred to nitrocellulose (Bio-Rad Laboratories, Hercules, CA) in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the membrane was blocked in 4% milk for 1 h at room temperature. Mouse monoclonal antibodies to FAS were purchased from BD Transduction Laboratories (Lexington, KY). Polyclonal antibodies to SREBP 1, PPARα, insulin receptor substrate (IRS) 2, and p65 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The SREBP1 antibody was raised against the N-terminal domain of SREBP1 and, thus, recognizes both the full-length mature protein (p125) and the N-terminal nuclear fragment of SREBP1 (p68). Acetylcoenzyme A carboxylase (ACC) α and β were detected as described previously (34). Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce, Rockford, IL).

Statistics

All data presented are mean ± SEM. Statistical analysis of studies comparing genotype and diet used a two-way ANOVA, with diet and genotype as variables, followed by all pair-wise multiple comparison procedures (Student-Newman-Keuls test). Statistical analysis used the SigmaStat Software for Windows version 2.03 (SPSS Inc., Chicago, IL). For studies comparing two groups, statistical analysis used the Student's *t* test.

Results

Experiment 1: analysis of metabolism in obese Mc4r−/− mice

Body weight and food intake data. Lep^{ob}/*Lep*^{ob} mice are temperature sensitive and exhibit signs of distress when housed in

wire-bottom cages at standard room temperatures (20–25 C); therefore, we maintained temperature at a constant 28 C. Female *Mc4r−/−* (n = 8), *Lep^{ob}/Lep^{ob}* (n = 10), and WT controls (n = 8), aged 2.5–3.5 months of age, were individually housed and fed the low-fat purified diet (D12450B, 10% kJ from fat) for 1 month. The mice were then acclimated to the metabolic chambers for 5–7 d. FM and FFM were measured 3 h after the end of the dark period on the first day of the experiment, after 3 d on the low-fat diet, and again after 3 d on the high-fat diet (D12451, 45% kJ from fat; Table 1). *Lep^{ob}/Lep^{ob}* mice had increased body weight due to more FM, whereas, for *Mc4r−/−* mice, increased body weight was due to more FM and FFM (FFM: WT, 14.8 ± 0.2 g; *Lep^{ob}/Lep^{ob}*, 15.9 ± 0.6 g; *Mcr4−/−*, 19.1 ± 0.6 g; $P < 0.01$ compared with WT and *Lep^{ob}/Lep^{ob}*; FM: WT, 1.9 ± 0.2 g; *Lep^{ob}/Lep^{ob}*, 20.3 ± 1.3 g; *Mcr4−/−*, 11.3 ± 1.1 g; $P < 0.01$ between all groups).

Energy intake data, total and adjusted for FFM, are shown in Table 1. Hyperphagia in *Mc4r−/−* mice was affected by dietary fat content, as reported previously using chow diets (16, 17). Although high-fat diets are normally expected to induce hyperphagia, we have observed an initial 20% reduction in energy consumption using diet D12451 in other experiments. Moreover, chronic exposure to diet D12451, unlike very high-fat diets, does not induce hyperphagia in longer term studies (35).

Comparison of EE in obese Mc4r−/− and Lep^{ob}/Lep^{ob} mice. In previous experiments examining EE of *Mc4r−/−* mice, recordings were limited to a short period in the light cycle (16). We were also not able to compensate for the greater metabolic activity of lean tissues compared with adipose (36), with VO_2 and EE data not adjusted for FFM. In the present study, we obtained EE data over several days, allowing us to examine the diurnal variation. VO_2 and EE exhibited a grossly normal circadian rhythm irrespective of genotype or diet (Fig. 1, A and B). As reported by others (37), obesity associated with leptin deficiency (*Lep^{ob}/Lep^{ob}*) was associated with higher EE adjusted for lean mass. However, obese *Mc4r−/−* mice appeared to have normal EE. Analysis using two-way ANOVA indicated significant effects of diet ($P < 0.05$) and genotype ($P < 0.05$), with least square means for VO_2 and EE listed in Table 2.

Comparison of the PRCF curves for VO_2 and EE reveal that, when fed the low-fat diet, the largest differences between strain occur in the upper quartile range (Fig. 2, A and C). After the introduction of the high-fat diet, the difference in the PRCF curves of VO_2 and EE for WT and *Mc4r−/−* mice became more noticeable, again in the upper quartile range (Fig. 2, B and D). This is consistent with the observation that the major differences in the increase of VO2 and EE of *Mc4r−/−* mice compared with WT and *Lep^{ob}/Lep^{ob}* mice occur during the lights-off phase (Fig. 1, C and D). This observation is also consistent with lower peak VO_2 and EE values for *Mc4r−/−* mice during the early phase of the dark cycle, observed upon careful observation of the diurnal rhythm (Fig. 1, A and B), and with the differences in diet-induced thermogenesis, which were greatest during the lights-off cycle (Fig. 1, C and D). This might indicate differences in activity-based EE or perhaps the thermic effect of feeding of *Mc4r−/−* mice compared with WT mice. The right shift of the curve of *Lep^{ob}/Lep^{ob}* mice compared with WT and *Mc4r−/−* mice occurs over the total curve, perhaps indicating changes in basal metabolic rate.

The lower metabolic rate of *Mc4r−/−* mice relative to *Lep^{ob}/Lep^{ob}* mice is an intriguing and unexpected observation. It is possible that the increased EE of obese *Lep^{ob}/Lep^{ob}* mice compared with obese *Mc4r−/−* mice represents an adaptive

TABLE 1. Body weight and food intake data for mice housed in metabolic chamber fed either an LF diet (10% kJ/fat, 20% kJ/protein, and 70% kJ/carbohydrate) or HF diet (45% kJ/fat, 20% kJ/protein, and 35% kJ/carbohydrate)

	WT	*Mc4r−/−*	*Lep^{ob}/Lep^{ob}*
Weight (g)			
Basal	21.5 ± 0.6	35.6 ± 1.1	40.8 ± 1.8
D 3, LF	22.5 ± 0.7	36.1 ± 1.1	42.1 ± 1.6
D 6, HF	22.1 ± 0.6	37.2 ± 1.1	42.4 ± 1.5
FM % total body weight			
Basal	10.1 ± 1.1	33.8 ± 1.5	47.3 ± 1.8
D 3, LF	11.4 ± 0.9	35.3 ± 1.2	51.3 ± 1.6
D 6, HF	11.0 ± 0.8	37.1 ± 1.3	51.4 ± 1.0
Energy intake per animal (kJ/d)			
LF diet	53 ± 4^a	63 ± 3^b	74 ± 4^c
HF diet	41 ± 2^a	74 ± 5^b	60 ± 2^c
Energy intake (kJ/g FFM·d)			
LF diet	3.6 ± 0.3^a	3.3 ± 0.2^a	4.7 ± 0.3^b
HF diet	2.8 ± 0.1^a	3.8 ± 0.2^b	3.9 ± 0.2^b
Two-way ANOVA:	kJ/d (*P*)		kJ/g FFM·d (*P*)
Genotype	<0.001		<0.001
Diet	<0.001		0.058
Genotype × diet	<0.005		<0.01

LF, Low-fat; HF, high-fat.

Mice were weighed and body composition determined by NMR on the first day of the study (basal), after 3 d on the LF diet (d 3), and after 3 d on the HF diet (d 6). Energy consumption data for mice housed in the metabolic chambers fed LF diet (10% kJ/fat, 20% kJ/protein, and 70% kJ/carbohydrate) or HF diet (45% kJ/fat, 20% kJ/protein, and 35% kJ/carbohydrate). Total energy consumption (kJ/d) was measured by weighing food every third day. Energy consumption was also adjusted for lean mass (kJ/g FFM·d), which was significantly higher in *Mc4r−/−* mice compared with WT and *Lep^{ob}/Lep^{ob}* mice. ^{a,b} Statistical analysis used two-way ANOVA and an all pair-wise multiple comparison procedure; all values within diet that do not share a *common letter* are significantly different ($P < 0.05$). Food intake on the LF and HF diets within genotype was significantly different in WT and *Lep^{ob}/Lep^{ob}* mice ($P < 0.01$).

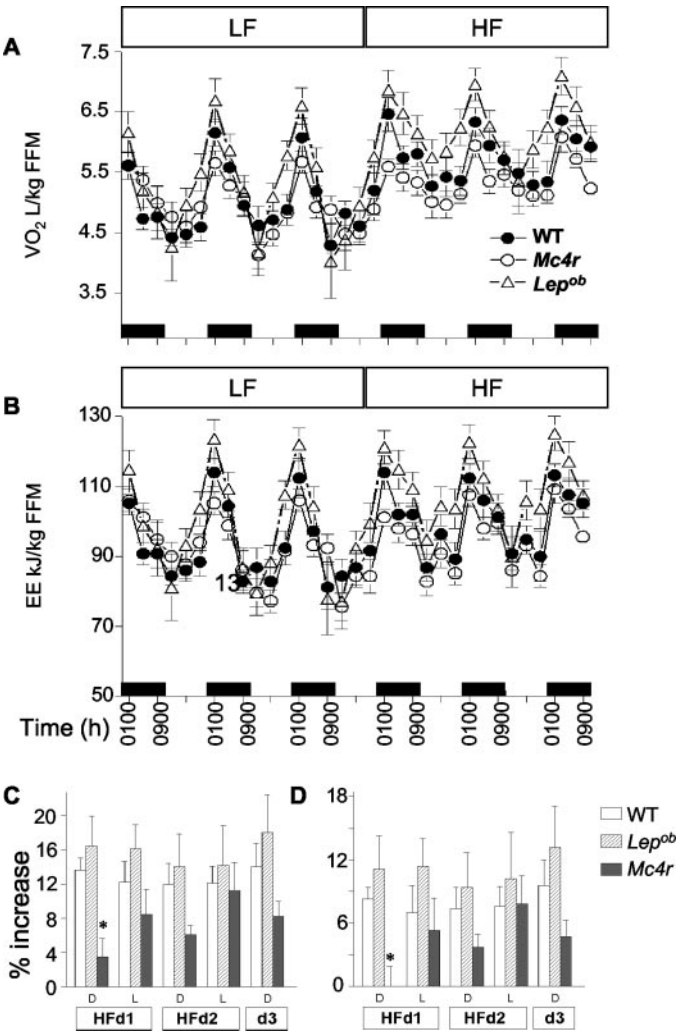

FIG. 1. Attenuated increase in VO_2 and heat of *Mc4r*−/− mice, relative to WT and *Lep*^{ob}/*Lep*^{ob} mice, in response to high-fat (HF) diet. Analysis of the data pooled as 4-h bins showed that the circadian rhythm in VO_2 (A) and EE (B) was not affected by genotype, with peak values occurring at the beginning of the dark phase. Introduction of the HF diet resulted in an increase in VO_2 and EE. To calculate the rate of increase, data from the light and dark periods were pooled and the percent increase calculated for each period for VO_2 (C) and EE (D). The percent increase in VO_2 and EE was lower in *Mc4r*−/− mice, especially in the dark period on d 1 of exposure to the HF diet. *, $P < 0.05$ compared with WT and *Lep*^{ob}/*Lep*^{ob}. LF, Low-fat.

response to weight gain that has been reported in humans (38). The attenuated increase in EE of obese *Mc4r*−/− mice could, therefore, indicate a role for this receptor in the increased EE associated with obesity. Alternatively, a threshold level of obesity required to increase EE might not have been achieved in *Mc4r*−/− mice, which were not as obese as the *Lep*^{ob}/*Lep*^{ob} mice (34–37% *vs.* 47–51% body fat, respectively; Table 1).

Substrate oxidation of female obese WT, Mc4r−/−, and Lep^{ob}*/Lep*^{ob} *mice fed purified low-fat and high-fat diets ad libitum.* For the 3 d on the low-fat diet, RER was stable and was not affected by genotype (mean RER over 3 d: WT, 0.984 ± 0.016; *Lep*^{ob}/*Lep*^{ob}, 0.988 ± 0.011; *Mc4r*−/−, 0.990 ± 0.021; Fig. 3A). Introduction of the high-fat diet was associated with a decline in the RER

TABLE 2. Least squares means of VO_2 and EE for *Mc4r*−/−, *Lep*^{ob}/*Lep*^{ob}, and WT controls during the lights-on and lights-off cycles

	WT	*Mc4r*−/−	*Lep*^{ob}/*Lep*^{ob}
VO_2 (liters/kg of FFM·h)			
Lights on	5.0 ± 0.2	4.9 ± 0.2	5.5 ± 0.1[a]
Lights off	5.7 ± 0.2	5.4 ± 0.2	6.0 ± 0.1[a]
EE (kJ/kg of FFM·h)			
Lights on	101 ± 4	97 ± 4	111 ± 3[a]
Lights off	116 ± 4	112 ± 4	124 ± 4[a]

The data represent the mean ± SEM of measurements for 3 d on the low-fat diet and 3 d on the high-fat diet.

[a] $P < 0.05$ compared with *Mc4r*−/−.

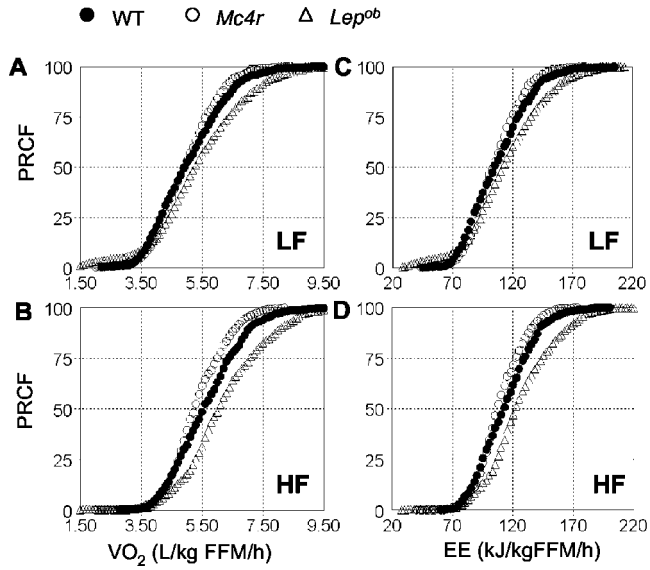

FIG. 2. PRCF of VO_2 (A, B) and heat (C, D) of WT, *Mc4r*−/−, and *Lep*^{ob}/*Lep*^{ob} mice on low-fat (LF) (A, C) or high-fat (HF) (B, D) diets. Each curve represents the cumulative frequency of 800-1200 data points for eight WT, eight *Mc4r*−/−, or 10 *Lep*^{ob}/*Lep*^{ob} mice.

irrespective of genotype. However, the mean RER over the 3 d on the high-fat diet was significantly higher in *Mc4r*−/− mice (RER: *Mc4r*−/−, 0.859 ± 0.012; *Lep*^{ob}/*Lep*^{ob}, 0.830 ± 0.011; WT, 0.804 ± 0.007; *post hoc* analysis: *Mc4r*−/− *vs.* WT, $P < 0.01$; *Mc4r*−/− *vs.* *Lep*^{ob}/*Lep*^{ob}, $P = 0.059$; *Lep*^{ob}/*Lep*^{ob} *vs.* WT, $P = 0.088$). The difference in the RER of *Mc4r*−/− mice fed the high-fat diet compared with both WT and *Lep*^{ob}/*Lep*^{ob} mice was maximal between 24 and 48 h after the introduction of the high-fat diet (Fig. 3A). During the dark period of d 2 on the high-fat diet, the RER of *Mc4r*−/− mice was significantly higher than WT and *Lep*^{ob}/*Lep*^{ob} mice ($P < 0.05$).

The ratio of RER to the FQ indicates the balance of nutrient intake and oxidation and must be balanced over the long term to maintain a constant body weight (39, 40). Energy stored as glycogen is rapidly depleted when compared with the far greater amount of energy stored in the form of TG. It has been proposed that, therefore, it is more important to maintain a balance between carbohydrate consumption and oxidation (39, 40). In these experiments, the RER to FQ ratio correlated with the change in weight on the high-fat diet (Fig. 3, C and D). On the low-fat diet, the RER to FQ ratio was greater than 1 irrespective of genotype, and all mice gained weight. On the high-fat diet, the RER to FQ ratio declined to

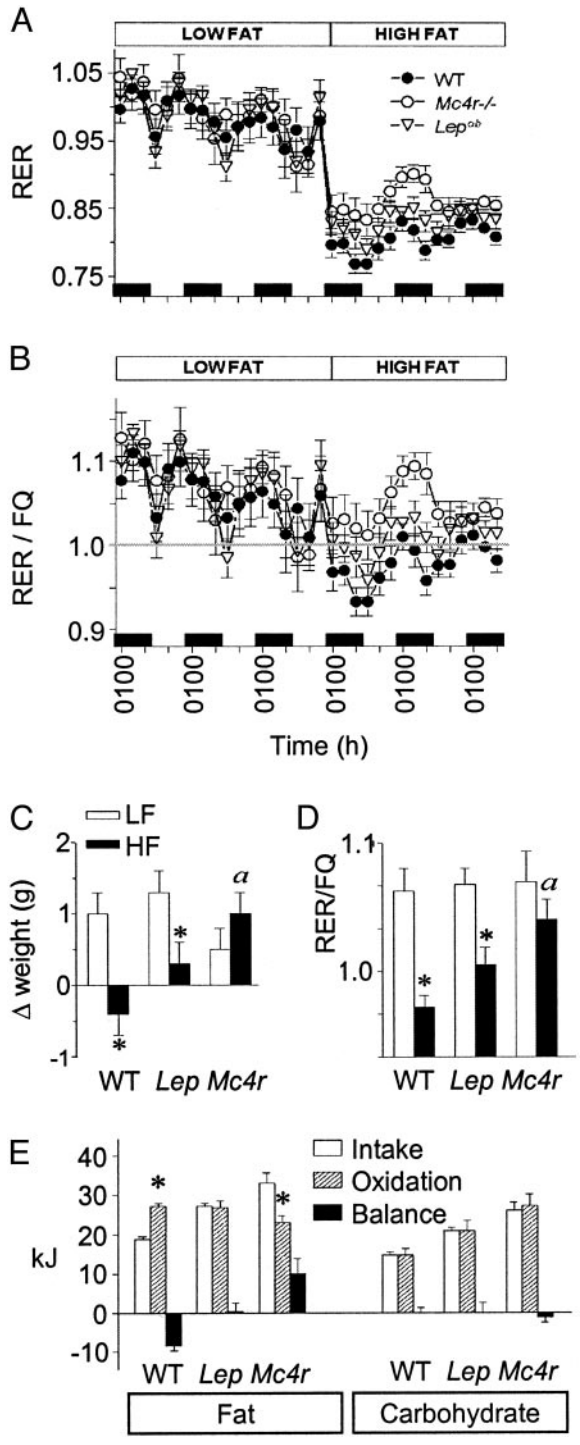

FIG. 3. Higher RER in *Mc4r−/−* mice, relative to WT and *Lep*^{ob}/*Lep*^{ob} mice, in response to high-fat (HF) diet is associated with a higher RER to FQ ratio, weight gain, and an imbalance in FA metabolism. (A) Analysis of the data as 4-h bins showed that RER ranged between 0.9 and 1.1 on the low-fa (LF) data and was similar between genotypes. Introduction of the HF diet resulted in a decline in the RER irrespective of genotype; however, the RER of *Mc4r−/−* mice was higher over the first 2 d. Comparison of the ratio of RER to FQ (RER/FQ) (B, D) with weight gain (C), and the balance (Bal.) of nutrient consumption (Int.) and oxidation (Oxidn) (E). Weight gain correlates with the RER to FQ ratio, with weight gain observed when the RER to FQ ratio is greater than 1 (analysis of weight gain by two-way ANOVA: genotype, $P = 0.185$; diet, $P = 0.018$; genotype × diet, $P = 0.06$; analysis of RER to FQ ratio by two-way ANOVA: genotype, $P = 0.071$; diet, $P < 0.001$; genotype × diet, $P = 0.179$; *, significantly different from LF within genotype, $P < 0.05$; *a*, significantly different from WT between genotype, $P < 0.05$). For the 3 d on the HF diet, weight gain correlated with the balance of fat consumption with FA oxidation (compare C with E). A negative balance of fat consumption and FA oxidation observed in WT mice was associated with weight loss, whereas a positive balance of fat consumption to FA oxidation observed in *Mc4r−/−* mice was associated with weight gain (*, significantly different from intake within genotype, $P < 0.01$). Carbohydrate consumption and oxidation were balanced irrespective of genotype.

less than 1 in WT and *Lep*^{ob}/*Lep*^{ob} mice for long periods. However, the RER to FQ ratio remained greater than 1 in *Mc4r−/−* mice fed the high-fat diet. *Mc4r−/−* mice gained significantly more weight than WT mice on the high-fat diet ($P < 0.01$).

The balance of substrate intake and oxidation during high-fat feeding was estimated by calculating the percent of total energy derived from FA oxidation (F%) using the RER (28) (Fig. 3E). The results of the analysis of fat and carbohydrate balance (kJ consumed − kJ oxidized) during the period on the high-fat diet indicate that carbohydrate intake and oxidation were balanced irrespective of genotype. In contrast, fat balance correlated with the RER to FQ ratio and weight gain data (compare Fig. 3E with Fig. 3, C and D), with only *Mc4r−/−* mice exhibiting a positive fat balance on the high-fat diet. Overall, the data are consistent with tightly regulated carbohydrate oxidation and consumption, with differences in weight gain correlating with the balance of fat consumption and oxidation.

Blood chemistries and liver lipid data of obese female WT, Mc4r−/−, and Lep^{ob}/*Lep*^{ob} *mice.* After the completion of indirect calorimetry experiments, all mice were returned to group housing (two per cage) for 4–6 wk on the low-fat diet. Mice were then either placed on the high-fat diet for 2 d, which was the period with the greatest differences in RER between the strains, or left on the low-fat diet (n = 6 per group). FM and FFM were measured after a 4-h fast, and tissues and sera were collected for analysis. Group-housed *Mc4r−/−* and *Lep*^{ob}/*Lep*^{ob} mice were hyperphagic compared with WT, irrespective of diet (data not shown). FM as a percent of total body weight did not change in WT mice and increased by 7% in *Mc4r−/−* mice and 11% in *Lep*^{ob}/*Lep*^{ob} mice (data not shown).

Blood chemistries for WT and obese *Mc4r−/−* and *Lep*^{ob}/*Lep*^{ob} mice are shown in Table 3. Both *Mc4r−/−* and *Lep*^{ob}/*Lep*^{ob} mice were hyperinsulinemic, although the increase was significant only for *Lep*^{ob}/*Lep*^{ob} mice ($P < 0.05$ compared with WT and *Mc4r−/−* mice). The lower fasting insulin in obese *Mc4r−/−* mice compared with obese *Lep*^{ob}/*Lep*^{ob} mice might be due to a more severe obesity or, alternatively, the increase in corticosterone associated with leptin deficiency. Hypercorticosteronemia is a significant factor in the insulin-resistant phenotype of leptin-deficient mice (41) and is not observed in *Mc4r−/−* mice (24). Diet had a significant effect on serum insulin (two-way ANOVA, $P < 0.01$), with a 2- to 3-fold increase in fasting insulin observed in *Mc4r−/−* and *Lep*^{ob}/*Lep*^{ob} mice on the high-fat diet compared with the low-fat diet. Blood glucose levels were normal in *Lep*^{ob}/*Lep*^{ob} mice

TABLE 3. Blood chemistries of WT, obese *Mc4r−/−*, and obese *Lep^{ob}/Lep^{ob}* mice

	WT		*Mc4r−/−*		*Lep^{ob}/Lep^{ob}*		Two-way ANOVA (*P*)
	LF	HF	LF	HF	LF	HF	
Insulin (pg/ml)	76 ± 31	96 ± 16	808 ± 369	1911 ± 554	2186 ± 489	6725 ± 976	Diet, <0.01 Genotype, <0.01
Glucose (mg/dl)	128 ± 3	131 ± 6	162 ± 9	172 ± 14	149 ± 7	137 ± 11	Diet, NS Genotype, <0.01
NEFA (mEq/liter)	0.54 ± 0.08	0.84 ± 0.18	0.80 ± 0.11	0.60 ± 0.07	0.84 ± 0.18	0.72 ± 0.11	Diet, NS Genotype, NS
TG (mmol/liter)	2.8 ± 0.6	2.2 ± 0.3	1.4 ± 0.2	1.7 ± 0.2	1.9 ± 0.3	2.3 ± 0.3	Diet, NS Genotype <0.05

LF, Low-fat; HF, high-fat; NS, not significant.

Mice were either fed a purified LF diet or fed a purified HF diet for 48 h. Mice were fasted for 4 h before the collection of serum samples. Statistical analysis used a two-way ANOVA, with diet and genotype as the independent variables.

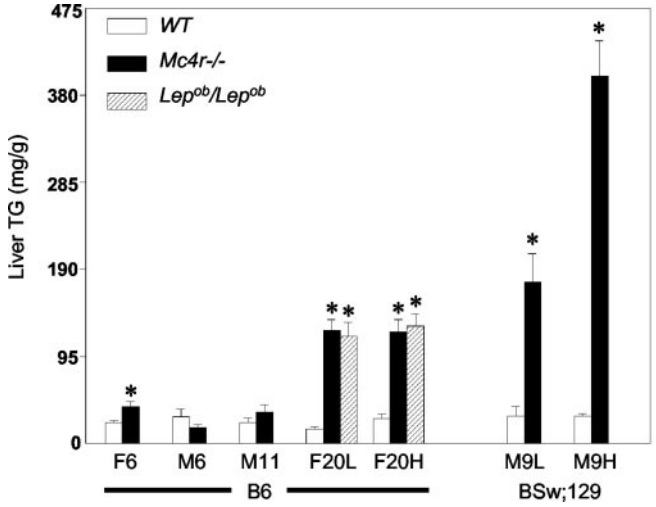

FIG. 4. Genetic background effects on the development of hepatic steatosis in *Mc4r−/−* mice. Preobese *Mc4r−/−* mice (6 and 11 wk old) in the B6 background do not exhibit hepatic steatosis, whereas preobese *Mc4r−/−* mice in the BSw;129 background exhibit severe hepatic steatosis at 9 wk of age. Liver TG content was measured in B6 *Mc4r−/−* mice in the B6 background at various ages (*left*) and in BSw;129 *Mc4r−/−* mice at 9 wk of age (*right*). F6, 6-wk-old female mice; M6, male 6-wk-old mice; M11, male 11-wk-old mice; F20L, 5-month-old obese female mice fed a purified low-fat diet; F20H, 5-month-old obese female mice fed a purified high-fat diet for 48 h; M9L, 9-wk-old BSw;129 mice fed low-fat diet; M9H, 9-wk-old BSw;129 mice fed high-fat diet. *, $P < 0.05$ compared with WT.

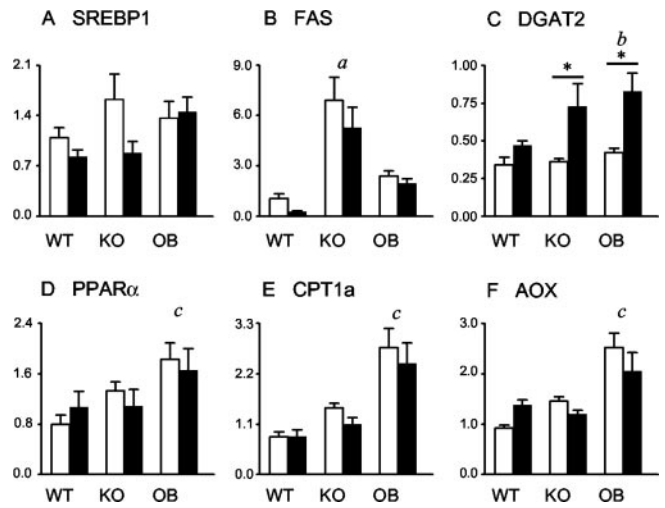

FIG. 5. Expression of genes involved in FA and TG synthesis (A–C) and FA oxidation (D, F) in liver of WT controls, obese *Mc4r−/−* (KO), and obese *Lep^{ob}/Lep^{ob}* mice (OB). Mice were fed the purified low-fat diet (*open bars*) or a purified high-fat diet for 48 h (*solid bars*). Blood chemistries for these mice are shown in Table 3. Statistical analysis used a two-way ANOVA (effects of genotype: *a*, $P < 0.001$ compared with WT and *Lep^{ob}/Lep^{ob}*; *b*, $P < 0.05$ compared with WT; *c*, $P < 0.05$ compared with WT and *Lep^{ob}/Lep^{ob}*; effects of diet: *, $P < 0.05$ compared with low-fat diet.

compared with WT, which agrees with earlier studies showing euglycemia in older animals (42, 43). *Mc4r−/−* mice exhibited a significant but small (30 point) increase in blood glucose ($P < 0.01$ compared with *Lep^{ob}/Lep^{ob}* and WT mice). Serum free FA levels were not significantly different between strain. On low-fat diet, serum TG levels were lower in *Mc4r−/−* mice compared with WT mice. Serum nonesterified FA (NEFA), TG, and glucose levels were not affected by diet. Liver TG content was increased in *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice to a comparable extent, with no effect of diet (Fig. 4)

Hepatic gene expression and protein levels in obese female WT, Mc4r−/−, and Lep^{ob}/Lep^{ob} mice. The expression of genes involved in lipogenesis and TG synthesis [SREBP1, FAS, and acylcoenzyme A:diacylglycerol acyltransferase (DGAT) 2; Fig. 5, A–C] and FA oxidation (PPARα, CPT1a, and AOX; Fig. 5, D and F) was examined using quantitative RT-PCR. ACC, FAS, SREBP1, PPARα, and IRS2 protein levels were also examined by Western blot analysis (Fig. 6).

Analysis of gene expression data using two-way ANOVA revealed that diet had no significant effect on the expression of lipogenic or oxidative genes. Genotype had no effect on the expression of SREBP1 mRNA (Fig. 5A) or protein levels (Fig. 6). In liver of *Mc4r−/−* mice, FAS mRNA levels were increased 7- to 10-fold compared with WT mice ($P < 0.001$) and 2- to 3-fold compared with *Lep^{ob}/Lep^{ob}* mice ($P < 0.001$) (Fig. 5B). The greater increase in FAS mRNA expression in *Mc4r−/−* mice compared with *Lep^{ob}/Lep^{ob}* mice correlated with changes in FAS protein levels, which were highest in *Mc4r−/−* mice (Fig. 6). The level of ACC protein, which catalyzes the first step in FA biosynthesis, was also increased in liver of *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice compared with WT mice (Fig. 6). DGAT2 mRNA, which encodes an enzyme involved in the final step of TG synthesis, was increased in *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice but was not significantly different on the low-fat diet (Fig. 5C). DGAT2 mRNA expression levels were increased by exposure to a high-

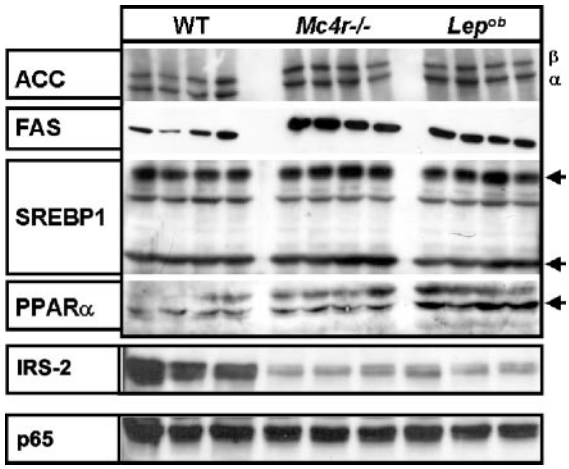

FIG. 6. Levels of ACC, FAS, SREBP1, PPARα, IRS2, and p65 (used as a loading control) proteins in liver of *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice compared with WT controls. An increase in FAS and ACC protein in *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice compared with WT controls was not associated with an increase in SREBP1, consistent with the mRNA data shown in Fig. 5. PPARα protein levels were increased in livers of *Lep^{ob}/Lep^{ob}* mice compared with *Mc4r−/−* and WT mice, which is also consistent with mRNA expression data shown in Fig. 5. The *upper band* in the SREBP1 blot represents the full-length form; the *lower band* represents the proteolytically cleaved transcriptionally active N terminus of SREBP1. For ACC, FAS, SREBP1, and PPARα, two samples from individual mice fed the low- or high-fat diets are shown. For IRS2 and p65, three samples from mice fed the low-fat diet are shown.

fat diet ($P < 0.05$), perhaps as the result of increased stimulation by insulin (44).

There was a 2- to 3-fold increase in the expression of genes involved in FA oxidation (PPARα, CPT1a, and AOX) in liver of *Lep^{ob}/Lep^{ob}* mice compared with WT and *Mc4r−/−* mice (Fig. 5, D–F). Analysis of PPARα protein levels by Western blot analysis confirmed that the increase in PPARα mRNA resulted in increased protein levels in *Lep^{ob}/Lep^{ob}* mice compared with both WT and *Mc4r−/−* mice (Fig. 6).

An age-dependent increase expression of PPARα mRNA in liver of obese *Lep^{ob}/Lep^{ob}* mice has been reported previously and is also observed in obese serotonin 2C receptor knockout mice (45). Our observation that the age-dependent increased expression of PPARα and PPARα-regulated genes does not occur in obese *Mc4r−/−* mice might indicate a role for this receptor in what could be an adaptive response to obesity. In addition, the increased expression of a lipogenic gene (FAS) and reduced expression of oxidative genes in the liver of *Mc4r−/−* mice compared with *Lep^{ob}/Lep^{ob}* mice correlate with differences in the thermogenic and substrate oxidation observed in these two obese strains in response to a high-fat diet (Figs. 1–3). The increased expression of FA oxidative genes appears to correlate with the robust thermogenic response and the higher rate of FA oxidation of *Lep^{ob}/Lep^{ob}* mice relative to *Mc4r−/−* mice. It is also possible that lipogenesis is reduced in obese *Lep^{ob}/Lep^{ob}* mice compared with obese *Mc4r−/−* mice.

The regulation of FAS mRNA expression is complex, involving numerous nuclear transcription factors (SREBP1c, liver X receptor, insulin-induced gene, and PPARγ) that respond to insulin, sterols, and carbohydrate levels (46–48). Hyperinsulinemia stimulates the expression of lipogenic genes in liver through SREBP1c and possibly PPARγ (49–51). Hyperinsulinemia also suppresses IRS2 transcription, leading to a reduced ability of insulin to suppress hepatic glucose output while simultaneously stimulating lipogenesis (51, 52). IRS2 protein levels were reduced to a similar extent in *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice, suggesting that hyperinsulinemia in both strains is associated with reduced signaling through IRS2 (Fig. 5).

The 10-fold increase in FAS mRNA in the liver of *Mc4r−/−* mice is interesting in that the increase was 3-fold greater than that observed in *Lep^{ob}/Lep^{ob}* mice, and it also does not appear to involve an increase inSREBP1 mRNA or protein (Figs. 5 and 6). Overall, a different pattern of the changes was observed in hepatic gene expression in obese *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice. In *Mc4r−/−* mice, a key lipogenic gene (FAS) is increased nearly 10-fold. In contrast, in *Lep^{ob}/Lep^{ob}* mice, there is an increase in the expression of a group of genes involved in FA oxidation (PPARα, AOX, and CPT1a). The increase in FAS mRNA in the liver of obese *Mc4r−/−* mice compared with obese *Lep^{ob}/Lep^{ob}* mice might be due to differences in nuclear SREBP1c, which were not measured in this study. Whether the changes in gene expression are due to a specific role for the MC4R in suppressing liver FAS expression and increasing PPARα activity or, alternatively, are due to local differences in liver metabolism in situations of obesity was not determined in this study. It is also important to note that the differences in hepatic gene expression between *Mc4r−/−* and *Lep^{ob}/Lep^{ob}* mice were not associated with changes in liver TG levels. This could indicate differences in the equilibrium of FAs between the liver and extrahepatic peripheral tissues.

Experiment 2: analysis of metabolism in preobese Mc4r−/− mice

FAS mRNA expression is not increased in preobese Mc4r−/− mice. Older *Mc4r−/−* mice exhibit an increase in FAS mRNA expression and a reduction in IRS2 protein that is similar to that reported in other mouse models of insulin resistance. We examined the extent to which the changes in hepatic gene expression were due to prolonged hyperinsulinemia, as opposed to a specific response to a reduction in MC4R activity, by comparing FAS mRNA expression in preobese *Mc4r−/−* and WT mice. Six-week-old female *Mc4r−/−* (n = 4), *Mc4r+/−* (n = 6), and *Mc4r+/+* (n = 5) mice were fasted overnight and then refed a purified low-fat diet for 3 h. Before refeeding, FFM and FM were measured by NMR. After refeeding, mice were anesthetized by brief exposure to CO_2 before euthanasia. The data shown are from female mice, similar results were observed in males.

At 6 wk of age, *Mc4r−/−* mice were significantly larger than their WT littermates; however, the difference was not due to an exclusive increase in FM (Fig. 7). Food intake over the 3-h refeeding period was not significantly different. Blood chemistry (insulin, leptin, glucose, and NEFA) were also not significantly different (Fig. 7). Liver TG content was significantly increased in preobese female *Mc4r−/−* mice (Fig. 4). However, the magnitude of the increase in liver TG in preobese female *Mc4r−/−* males was mild when compared with that observed in older obese *Mc4r−/−* mice. Moreover, liver TG levels were normal in male *Mc4r−/−* mice at 6 wk and 11 wk of age. Liver

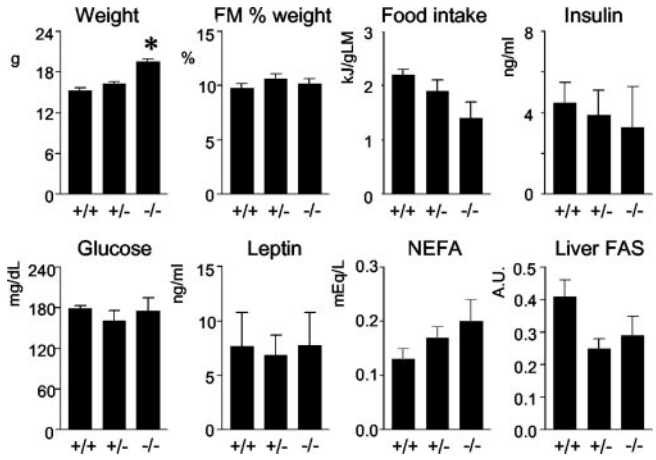

FIG. 7. Preobese *Mc4r*−/− and *Mc4r*+/− mice in the B6 background are not hyperinsulinemic or hyperglycemic and have normal levels of leptin and NEFA. Liver FAS mRNA expression is not significantly different in the absence of hyperinsulinemia. Hepatic steatosis and increased FAS mRNA and protein expression observed in older B6 *Mc4r*−/− mice (Figs. 4–6) is, therefore, probably secondary to obesity-induced hyperinsulinemia.

FAS mRNA expression was not significantly different in preobese 6-wk-old female *Mc4r*−/− mice (Fig. 7) or in 6-wk or 11-wk-old male *Mc4r*−/− mice (data not shown). Overall, the data suggest that the development of hepatic steatosis in *Mc4r*−/− mice in the B6 background is likely to be secondary to hyperinsulinemia associated with obesity.

Preobese Mc4r−/− *in the BSw;129 background can exhibit hepatic steatosis without fasting hyperinsulinemia.* Genetic background can have a marked effect on the genotype of transgenic, knockout mice and spontaneous mutant mice (53, 54). To determine whether genetic background has an effect on the development of insulin resistance, we examined the phenotype of BSw;129 *Mc4r*−/− mice. We used male BSw;129 *Mc4r*−/− mice aged 8 wk that had been fed the purified low-fat diet or a purified very high-fat diet (D12492, 60% kJ from fat) for 5 d. FFM was not significantly different in BSw;129 *Mc4r*−/− and BSw;129 *Mc4r*+/+ mice at this age and in this strain (data not shown). On the low-fat diet, FM was similar in BSw;129 *Mc4r*−/− and BSw;129 *Mc4r*+/+ mice (FM as a percent of total body weight: BSw;129 *Mc4r*−/−, 18.2 ± 1.1%; BSw;129 *Mc4r*+/+, 16.0 ± 1.5%). On the high-fat diet, FM was significantly higher in BSw;129 *Mc4r*−/− mice compared with BSw;129 *Mc4r*+/+ mice (FM as a percent of total body weight: BSw;129 *Mc4r*−/−, 23.6 ± 2.3%; BSw;129 *Mc4r*+/+, 18.0 ± 1.8%).

Liver and serum were collected from mice that had been fasted for 4 h. Unlike 6-wk and 11-wk-old *Mc4r*−/− mice in the B6 background, a marked increase in liver TG was observed in BSw;129 *Mc4r*−/− mice compared with WT Bsw;129 mice (Fig. 4). Liver TG levels in BSw;129 *Mc4r*−/− mice that were fed the high-fat diet were also significantly increased compared with the levels observed in BSw;129 *Mc4r*−/− mice fed the low-fat diet ($P < 0.01$). Hepatic steatosis in BSw;129 *Mc4r*−/− mice does not appear to be secondary to obesity or hyperinsulinemia. The accumulation of TG in preobese BSw;129 *Mc4r*−/− mice indicates defects in hepatic FA uptake, FA oxidation, and/or TG secretion in this strain.

The large increase in liver TG would be predicted to be associated with fasting hyperinsulinemia and hyperglycemia. However, this was not the case in most of the BSw;129 *Mc4r*−/− mice examined. Overall, two groups of BSw;129 *Mc4r*−/− mice could be distinguished based on fasting insulin and the expression of SREBP1 and FAS mRNA in liver. Data from a representative selection of individual BSw;129 *Mc4r*−/− and BSw;129 *Mc4r*+/+ mice are shown in Fig. 8.

The majority of BSw;129 *Mc4r*−/− mice (nine of 11 mice) had fasting serum insulin levels in the normal range (562 ± 67 pg/ml compared with 478 ± 31 pg/ml for BSw;129 *Mc4r*+/+ mice, $P = 0.3$). Because diet did not have a significant effect on serum insulin, the data from the low-fat and high-fat diet groups were pooled for this analysis. The BSw;129 *Mc4r*−/− mice with normal insulin levels also had normal expression of SREBP1 and FAS mRNA (data not shown), which was also observed in preobese *Mc4r*−/− mice in the B6 background. Two BSw;129 *Mc4r*−/− mice, which happened to be from the group fed the low-fat diet, did have fasting hyperinsulinemia, with insulin levels of 2362 and 1417 pg/ml. Fasting hyperinsulinemia did not correlate with

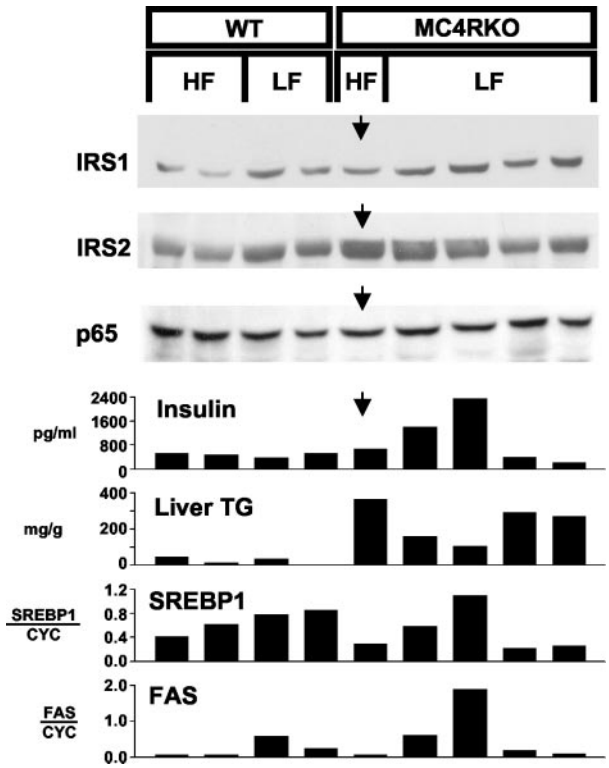

FIG. 8. Western blot of IRS1 and IRS2 protein, serum insulin, liver TG, and hepatic SREBP1 and FAS mRNA expression for individual preobese BSw;129 *Mc4r*−/− and BSw;129 *Mc4r*+/+ mice. In this figure, it is apparent that IRS2 protein levels are not reduced in preobese BSw;129 *Mc4r*−/− mice. In addition, hepatic steatosis in some preobese BSw;129 *Mc4r*−/− mice is associated with fasting insulin in the normal range. Overall, fasting insulin correlates with hepatic SREBP1 and FAS mRNA but not with liver TG. This suggests that the development of hepatic steatosis in this model occurs before the development of fasting hyperinsulinemia. Western blots of IRS1, IRS2, and p65 (a loading control) are compared with serum insulin, liver TG, and hepatic SREBP1 and FAS mRNA expression for individual animals. The *arrow* points to a preobese BSw;129 *Mc4r*−/− mouse fed a purified high-fat (HF) diet and is included for reference. LF, Low-fat diet; CYC, cyclophilin B.

higher levels of liver TG compared with other BSw;129 *Mc4r*−/− mice, ranging from 107–162 mg/g (Fig. 8). However, SREBP1 and FAS mRNA levels were higher in the two hyperinsulinemic BSw;129 *Mc4r*−/− mice, with the increase in SREBP1 and FAS mRNA proportional to insulin levels (Fig. 8).

On the low-fat diet, fasting blood glucose levels were moderately increased in BSw;129 *Mc4r*−/− mice compared with BSw;129 *Mc4r*+/+ mice (blood glucose: BSw;129 *Mc4r*−/−, 129 ± 8 mg/dl; BSw;129 *Mc4r*+/+, 100 ± 6 mg/dl). The very high-fat diet increased blood glucose 20–30 points in both BSw;129 *Mc4r*−/− and BSw;129 *Mc4r*+/+ mice (BSw;129 *Mc4r*−/−, 165 ± 8 mg/dl; BSw;129 *Mc4r*+/+, 121 ± 6 mg/dl). Analysis using two-way ANOVA indicates that the effects of diet and genotype on fasting blood glucose are significant ($P < 0.01$); there was no effect of genotype on the effect of diet (genotype X diet, $P = 0.264$). The mild increases in fasting glucose associated with the high-fat diet and loss of MC4R signaling might indicate increased hepatic glucose output or insulin resistance in extrahepatic tissues.

IRS2 protein levels are normal in steatotic livers of preobese BSw;129 Mc4r−/− mice. In *Lep*^{ob}/*Lep*^{ob} and lipodystrophic mice, hepatic steatosis is associated with hepatic insulin resistance, with a reduction in IRS2 protein and an increase in SREBP1 and FAS (51, 55). So far, our data indicate that a dramatic increase in liver TG can occur in BSw;129 *Mc4r*−/− mice that is not necessarily dependent on increased SREBP1 or FAS activity. To examine whether the increase in hepatic SREBP1 and FAS mRNA observed in the two hyperinsulinemic BSw;129 *Mc4r*−/− mice is also associated with reduced insulin signaling, we examined IRS1 and IRS2 protein by Western blot analysis (Fig. 8). Overall, IRS1 and IRS2 protein levels appeared to be increased, not reduced, in liver of BSw;129 *Mc4r*−/− mice compared with BSw;129 *Mc4r*+/+ mice. Hepatic steatosis in preobese BSw;129 *Mc4r*−/− is thus distinguishable from that observed in *Lep*^{ob}/*Lep*^{ob} and lipodystrophic mice in that it is not necessarily associated with elevated FAS or reduced IRS2.

Discussion

Overall, the major findings of these experiments fall into two areas. First, obese *Mc4r*−/− mice have a significant reduction in EE adjusted for FFM when compared with obese *Lep*^{ob}/*Lep*^{ob} mice. The differences in EE of obese *Lep*^{ob}/*Lep*^{ob} mice compared with obese *Mc4r*−/− mice correlate with changes in the expression of genes involved in FA oxidation in liver. Second, we have observed a significant effect of genetic background on the development of hepatic steatosis in *Mc4r*−/− mice. In the majority of BSw;129 *Mc4r*−/− mice, the 10- to 20-fold increase in liver TG was not associated with fasting hyperinsulinemia or increased FAS mRNA expression. The results from experiment 2 suggest that the development of hepatic steatosis in *Mc4r*−/− can in some circumstances occur independently of an increase in lipogenic gene expression and could involve defects in FA uptake and oxidation or TG secretion. In contrast, hepatic steatosis in *Lep*^{ob}/*Lep*^{ob} and lipodystrophic models of insulin resistance develops rapidly and is thought to be at least partially due to increased transcription of lipogenic genes in response to hyperinsulinemia (51, 55, 56).

The comparison of calorimetry data between lean and obese strains can be difficult to interpret. Adjusting EE for FFM assumes that FM has a negligible metabolic rate. However, it is important to note that the *Mc4r*−/− and *Lep*^{ob}/*Lep*^{ob} mice used for indirect calorimetry in the present study were both obese. If FM has a significant contribution to total-body EE, we would have expected differences in EE to correlate with the differences in FM observed between genotypes (WT < *Mc4r*−/− < *Lep*^{ob}/*Lep*^{ob}). This was not the case, and in fact, EE appeared to be reduced in *Mc4r*−/− mice not only compared with *Lep*^{ob}/*Lep*^{ob} mice but also compared with WT controls.

The mechanism explaining the increase in EE of *Lep*^{ob}/*Lep*^{ob} mice remains unclear, although an increase in the expression of FA oxidative genes in liver might be a contributing factor. Hyperphagia is one possible cause for the increased metabolic rate of *Lep*^{ob}/*Lep*^{ob} mice compared with WT and *Mc4r*−/− mice on the purified low-fat diet. However, it is interesting to observe that on the high-fat diet, where energy consumption was equal, *Lep*^{ob}/*Lep*^{ob} mice were still hypermetabolic compared with *Mc4r*−/− mice. One possibility is that the increased metabolic rate in obese *Lep*^{ob}/*Lep*^{ob} mice might at least partially represent a long-term adaptation to obesity similar to that reported in some clinical studies of obese subjects (38).

Finally, the diabetic phenotype of *Mc4r*−/− mice in the B6 background is very mild when compared with that reported for mice in a mixed 129;B6 background. This observation is perhaps not surprising when the effects of genetic background on the development of DM2 in *Lep*^{ob}/*Lep*^{ob} mice is taken into consideration. *Lep*^{ob}/*Lep*^{ob} mice in the B6 background are able to maintain normal glucose levels by increasing insulin output from an increased population of β cells, whereas *Lep*^{ob}/*Lep*^{ob} mice in other backgrounds develop severe DM2 (43, 54).

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